SZENT ISTVÁN UNIVERSITY

PH.D. THESIS

DEVELOPMENT OF GENETIC TOOLS FOR MOLECULAR BIOLOGY ANALYSIS OF THE THERMOACIDOPHILIC *THERMOPLASMA ACIDOPHILUM* MODEL ORGANISM

Erzsébet Baka

GÖDÖLLŐ

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Ph.D. School

Name: Ph.D. School of Environmental Sciences

Discipline: Environmental Sciences

Leader of School: Csákiné Dr. Erika Michéli, Ph.D.
Professor, head of department
Szent István University
Faculty of Agricultural and Environmental Sciences
Institute of Environmental Sciences
Department of Soil sciences and Agrochemistry

Supervisors: Dr. József Kukolya
Senior Scientist, head of department
Central Environmental and Food Science Research Institute

Department of Microbiology

Dr. Balázs Kriszt
Associate professor, head of department
Szent István University
Faculty of Agricultural and Environmental Sciences
Institute of Environmental and Landscape Management
Department of Environmental Protection and Safety

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Approval of School Leader Approval of Supervisors

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1. BACKGROUND

Since their discovery Archaea gained a special role in research, since they harbour features resembling to both prokaryotic and eukaryotic cells. Initial research suggested that these microorganisms could only strive in extreme conditions (in example highly acidic, hypersalinic or hyperthermal environments). Nowadays, this picture changed a lot and we now know that these microorganisms are omnipresent on our planet and locally represent up to 20% of the whole microbiome. After discovery, researchers assumed that the common ancestor group of all cells was identified, providing answers to open questions related to evolution of microorganisms, since Archaea adapted to extreme environments, typical for the time when life appeared on our planet. In addition, Archaea have raised the interest of biotechnologists because their enzymes exhibit adaptation to extreme environments (such as temperatures well above and below the 37 °C typical for human physiology), which make them versatile tools in industrial and medical applications.

Morphology and main metabolic pathways of Archaea are similar to Bacteria, though some metabolic pathways like methanogenesis are unique in this kingdom. Again other pathways responsible for genetic processing and protein turnover metabolism largely resemble those of eukaryotes, and enzymatic machinery in Archaea often exhibits less complexity, providing simpler models for investigation. For this reason research focus was lately shifting towards this group of microorganisms.

I chose a special Archaeon, *Thermoplasma acidophilum*, as the subject of my research. It thrives at temperatures between 40 and 60 °C in an environmental pH between 1 and 4, and surprisingly entirely lacks a cell wall. A few years after its discovery in 1970 the organism was thought to be the ancestor of eukaryotic cells, but the theory was dropped based on the results of its genome project frinished in 2000, exploring a number of unique characteristics of *T. acidophilum*. Nowadays *T. acidophilum* has an important role in the exploration of structure and function of eukaryotic protein and enzyme complexes, by providing simplified model complexes with high similarity to their eukaryotic counterparts. Significant insights into eukaryotic proteolytic processes were gained by studying the proteasome and associated factors in *T. acidophilum*, highlighting the clinical relevance of this research. Additionally, the organism's small cellular size and the lack of a cell wall

make it ideal for electron-cryotomographic investigations, which aim to understand spatial organization of the cellular proteome. Despite availability of an assembled genome and intensive classical proteomic research, further work with this microbe has proven to be considerably difficult. Clonal work was cumbersome and despite continuous and great efforts, development of genetic tools for manipulation of the members of the *Thermoplasma* taxon posed a number of yet unsolved challenges. Genetic tools however would provide a milestone for deepened investigation of structure and function of those proteins, which escaped thorough characterization, because they are not suitable for heterologous expression, or if expressed in *Escherichia coli,* they often do not exhibit biological activity. Of all identified proteins 29% are of unknown function and do not resemble any other known protein, and further 16% have not been conclusively identified yet and are thus termed as hypothetical unique proteins. Genetic tools would greatly aid to produce sufficient quantities of these proteins in its native host for further investigation.

2. AIMS

The aim of my work was to develop the missing basic genetic tools for the *T. acidophilum* model organism.

During development genetic tools I planned to create and generate the following elements:

- Routine solid cultivation of the organism; an essential element of clonal work to generate genetically homogenous cell lines.
- Separation of wild type and genetically manipulated cells, development of selection markers and resistance against antibiotics that can tolerate the extreme culturing conditions.
- Creation of vectors and constructs suitable for transfer of genetic elements, which contain the appropriate selection marker gene.
- Development of a transformation method to introduce nucleic acid constructs into the organism effectively and reproducibly.

Fulfilment of these aims will open new gates to *Thermoplasma* research, potentially accelerating investigations of unknown target proteins, and analysis of 6 proposed target proteins by knock-out mutagenesis currently prioritized by the Max Planck Institute of Biochemistry, which strongly supported my work. Besides these specific targets, the new genetic tools could be used to analyse structure and activity of low-abundant, labile large molecule complexes.

3. MATERIAL AND METHODS

Creation of prerequisites of clonal work: cultivation in liquid and on solid medium

The liquid medium for cultivation of *T. acidophilum* was prepared according to literature, and modified in order to increase growth speed of the organism. The amount of industrial yeast extract was increased four-fold and supplemented with self-made yeast vitamin. The yeast vitamin was prepared from commercially available baker's yeast biomass: 10 g baker yeast was added to 100 ml 0.5 M sulfuric acid, sonicated twice for 1 min at 40% output, then incubated at 58 °C for 3 h. After, cellular debris was removed by centrifugation (20 min at 4600 ×g, 4 ^{o}C), and the supernatant was sterile-filtered. I added 50 ml yeast vitamin per liter liquid medium. A Gelrite-based solid medium was prepared for solid cultivation according to literature. The amount of casamino acids, industrial yeast extract, and Gelrite (solidification agent) was doubled and the medium was supplemented by 50 ml/L yeast vitamin. cultivations were performed at 58 ^{o}C, cultures on solid medium were kept in a sealed jar to avoid excessive evaporation.

Antibiotic sensitivity tests

Antibiotics were purchased from Sigma-Aldrich Ltd. (Budapest, Hungary) for sensitivity tests, and stock solutions were prepared according to the manufacturer's instructions. Sensitivity of T. acidophilum was tested for each antibiotic at the suggested concentration range. Minimal inhibitory concentration (MIC) was determined after 4 days (96 hours).

Development of vector constructs

During development of vector constructs standard methods (after Sambrook) were combined with a modified protocol of the overlapping-megaprimer method. Genomic DNAs were isolated using MoBio UltraClean Microbial DNA Isolation Kit, or by phenol-chloroform and Na-perchlorate DNA isolation. Plasmids DNAs were isolated using QIAprep Spin Miniprep Kit and QIAGEN Plasmid Mega Kit according to the manufacturers' instructions. PCR reactions were performed using *Taq* and *Pfu* polymerase and optimized for expected product size and annealing temperature of the primers. Site-directed mutagenesis was done using QuikChange Site-Directed Mutagenesis Kit according to the manufacturer's instructions, or by overlapping PCR

mutagenesis. Prior to application all vector constructs were analysed by restriction digestion and sequencing.

Dialysis-based transformation *T. acidophilum*

The pH of 30 ml ultrapure water in a Petri dish was adjusted with dry ice to 4.5 before preheating it to 58 °C, yielding the dialysis buffer. Further steps were carried out at 58 °C. 200 µl *T. acidophilum* cell culture with an OD600 of 0.8-1.0 was gently pipetted onto a dialysis membrane (Millipore VSWPO2500 - Millipore GmbH, Germany) floating on the dialysis buffer. When the pH of the cell suspension increased to 3.5, generally after 60 min of dialysis, 10–20 µl plasmid DNA (concentration: 0,5–2 µg/µl) in ultrapure water was added to the cells and the mixture was incubated for an additional 60 min. Subsequently, cells were transferred into 1 ml fresh growth medium without selection agent and allowed to regenerate at 58 °C for 12 h. Regeneration was perfomed in a thermomixer (Eppendorf). For selection of transformants, 200 µl aliquots of the cell suspension were spread on antibiotic-containing solid medium and incubated at 58 °C in a micro-aerobic and humid environment until colonies appeared, usually after 12 days. At each transformation a positive growing control was applied treated the same way as the transformation samples but finally I transferred into antibiotic-free media. Furthermore, at each transformation a negative control was adapted to which plasmid DNA was not added but finally incubated in antibiotic containing media.

Confirmation of genetic exchange – analysis of transformants

After transformation and selection, transformants were parallely analysed by antibiotic resistance tests and molecular methods. For investigation of plasmid maintenance, plasmids were isolated from resistant cell lines, examined by agarose gel electrophoresis and nanophotometer, and re-transformed to *E. coli* competent cells. Newly isolated plasmids from *E. coli* were analysed by restriction digestion and sequencing. For investigation of genome integration, sequence analysis of the product created by primers homologous to regions outside of the target genomic region was performed, besides verifying the presence of resistant genes by gene-specific PCR reactions.

4. RESULTS AND DISCUSSION

3.1 *Thermoplasma* medium optimization and cultivation on solid medium

During my work I experienced that *T. acidophilum* responded positively to an increased amount of industrial yeast extract (**Figure 1.**). A twofold (2 g) increase slightly, a fourfold increase greatly enhanced the growth speed of the cells. Based on scientific literature it was known the way of preparation of the yeast extract influences the growth promoting effect. The yeast vitamin was prepared from bakers' yeast (*Saccharomyces cerevisiae*) by acidic hydrolysis at elevated temperature. The amount of yeast vitamin is shown as dry cell mass (DCM). Results are displayed in **Figure 1**. Fastest growth was achieved with 4 g industrial yeast extract and 0.5 g yeast vitamin. (1. figure, purple curve).

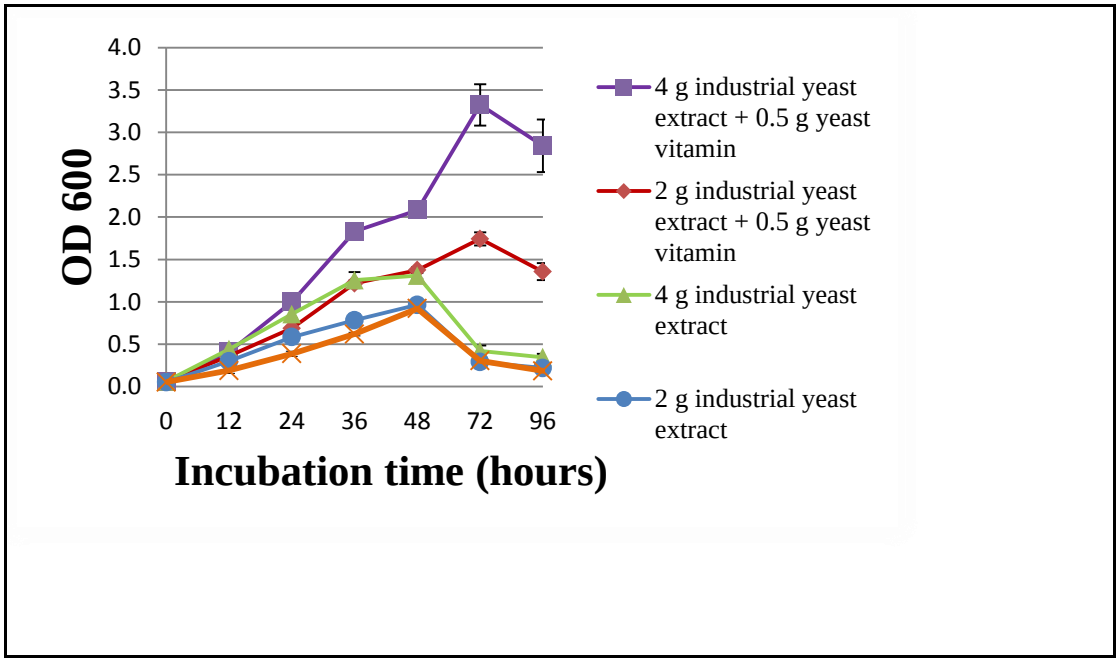

FIGURE 1. GROWTH OF *T. ACIDOPHILUM* IN THE PRESENCE OF DIFFERENT AMOUNT OF INDUSTRIAL YEAST EXTRACT AND 0.5 G YEAST VITAMIN

During development of genetic tool the possibility of clonal selection is crucial to yield genetically homogenous cell lines. Based on previous knowledge I started to develop such a solid medium on which *T. acidophilum* cells could form colonies in shorter time and the solidification agent could tolerate the high temperature and low

pH required by the cell growth. In all cases the solidification agents: gelrite (Sigma Aldrich Ltd), silica (Sigma Aldrich Ltd.), phytagele (Sigma Aldrich Ltd.), noble-agar (Becton Dickinson Hungary Ltd) and seakem gold agarose (Lonza Group Ltd) were able to tolerate the high temperature and the low pH, but *T. acidophilum* did not grow on seakem gold agarose- and noble-agar-based solid medium, while cells from colonies grown on silica- and phytagele- based solid media did not grow in liquid medium after solid cultivation. gelrite-based solid medium was based on previous Japanese research, and further modified to decrease the original 30 days incubation time. In order to increase the hardness against mechanical force the Gelrite amount was increased to 1,2% and $CaCl_2$ amount to 10 mM. Moreover, I applied the formerly developed yeast vitamin in a final concentration of 5% (w/v). Cultivation on gelrite-based solid medium was performed in a sealed jar to maintain steady temperature and humidity. With these modifications the organism was able to form yellow-brown colonies of 1-3 mm diameter within 12 days of incubation (**Figure 2.**).

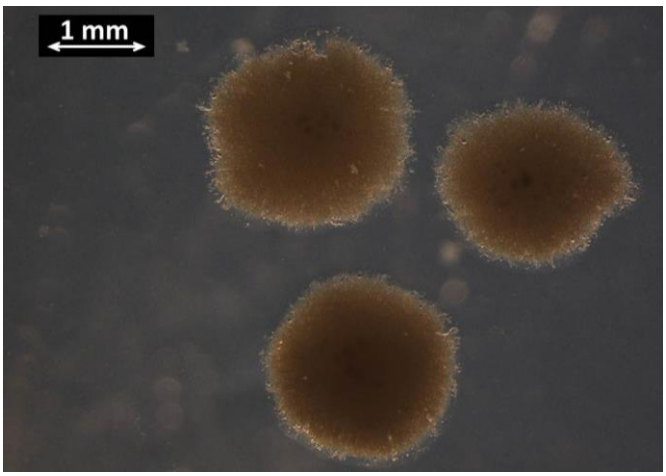

FIGURE 2. *T. ACIDOPHILUM* COLONIES ON GELRITE-BASED SOLID MEDIUM ON THE 12TH DAY OF INCUBATION

Cells from colonies grown on gelrite-based solid medium sustained growth when transferred into liquid medium. After transformation gelrite-based solid medium was applied supplemented by the appropriate antibiotic.

NEW SCIENTIFIC RESULT (I. thesis, based on results of 3.1. chapter): The modified Gelrite-based solid medium is suitable for accelerated routine cultivation of *Thermoplasma acidophilum*, thus it is appropriate for clonal selection, generation of genetically homogeneous cell lines.

3.2 Antibiotic sensitivity tests and selection marker genes

During development of genetic tools it is a crucial element to divide genetically manipulated cells from wild type cells. The minimal inhibitory concentration (MIC) was determined for 9 antibiotic involved in the experiments after 4 days (96 hours) according to the generation time of *T. acidophilum*. Results of the antibiotic sensitivity tests are illustrated by **1. table**.

1. Table. Results of antibiotic sensitivity tests

	Antibiotic	**MIC**
Nucleic acid synthesis inhibitors		
	Coumermycin A	10 ng/ml
	Novobiocin	**10 ng/ml**
	Rifampicin	**1 µg/ml**
Protein synthesis inhibitors		
	Anisomyicin	-
	Apramycin	-
	Erythromycin	100 µg/ml
	Chloramphenicol	250 µg/ml
	Thiostrepton	200 µg/ml
	Tetracycline	-

Later novobiocin and rifampicin were applied in my experiments. Novobiocin belongs to the amino-coumarins inhibiting DNA gyrase and the organism was reported to be highly sensitive for the gyrase blocking novobiocin. Rifampicin inhibits the transcription via binding to DNA-dependent-RNA-polymerase. Surprisingly in my *in vivo* experiments *T. acidophilum* showed great sensitivity for rifampicin, whereas according to previous research RNA polymerase of *T. acidophilum* was reported to be resistant in *in vitro* analyses.

A given antibiotic can be only used as selection marker in a target organism, if a selection marker gene can be assigned to it which can be expressed and cause resistance. In case of novobiocin a mutant *gyrB* gene causing novobiocin resistance was reported. Novobiocin-based vector constructs carried either a Nov^R mutant *gyrB* gene from HO-62N1C strain, a version modified by site-directed mutagenesis (Nov^R *gyrB*_A136H), besides an artificial *gyrB* gene showing total homology to Nov^R *gyrB*_A136H designed by codon degeneration in a way that maximally 5 base pair long DNA stretches were identical with the original nucleotide sequence. In my

experiments I used the *arr2* gene from *Pseudomonas aeruginosa* that inactivates rifampicin via ADP- ribosylation.

NEW SCIENTIFIC RESULT (II. thesis, based on results of 3.2. chapter): Based on the antibiotic sensitivity test erythromycin in a concentration of 100 µg/ml, chloramphenicol at 250 µg/ml, coumermycin A at 10 ng/ml, novobiocin at 10 ng/ml, rifampicin at 1000 ng/ml and thiostrepton at 200 µg/ml are suitable for selection of transformats.

3.3 Vector constructions

Essential elements of genetic tools are vector constructs with new genetic information, to be introduced into the target organism.

3.3.1 Shuttle vectors

Shuttle (or bifunctional) vectors are able to replicate in two different organism independently from the chromosome. These vectors were designed for purposes of future protein overexpression. In this case the shuttle vectors can be maintained in *E. coli* and *T. acidophilum*, since they carried the origins of replication for both organisms (the initiation point of replication, which carries an organism specific sequence) and resistant marker genes. These vectors have three major elements: (a) the base of the vector from pRSF Duet *E. coli* cloning vector, which bore *E. coli* specific RSF ori and Kan^R (kanamycin resistant gene), (b) T. *acidophilum* specific selection marker gene and (c) 6106 bp *Nco*I fragment from the only known *T. acidophilum* endogenous plasmid, pTA1 (position: 13534-3916) which presumably harbours a *T. acidophilum*-specific ori region. The pTA1 plasmid was isolated from a Japanese *T. acidophilum* strain, HO-122 with QIAGEN Plasmid Mega Kit. The two shuttle vectors were different in resistance gene and promoter used, and named as follows: pSTA - *T. acidophilum* Nov^R *gyrB* gene (Genbank accession number: KC710297), pSTRif - Rif^R *arr2* gene (**Figure 3.**). For conferring continuous and high antibiotic resistance such promoters were chosen and cloned, which were either presumably constitutive (Ta1137 – gene responsible for protein folding - Nov^R *gyrB*) or their gene product is expressed in large amounts (Ta1288 – proteasome α-subunit gene - *arr2*). Prior to transformation of *T. acidophilum* vectors were verified by sequencing.

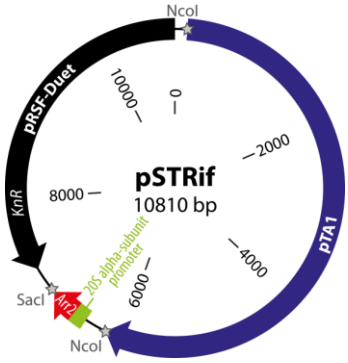

FIGURE 3. PSTRIF SHUTTLE VECTOR MAP

3.3.2 Integrative vectors

The integrative vectors that were built for homologous recombination (integration of genetic information into the chromosome) purposes did not contain the ori regions for independent replication in the target organism.

Base of the integrative vectors was pUC18, an *E. coli* specific plasmid. The truncated gyrase operon including the *gyrB* (gyraseB) and the partial *gyrA* (gyraseA) from the wild type *T. acidophilum* (DSM 1728) was cloned between the *Nde*I and *Eco*RI site of the pUC18 plasmid. The integrative vectors differed only by the resistance gene. The pDTA plasmid (Genbank accession number: KC710295) (**Figure 4.**) bore the mutant *gyrB*. The pNTA vector carried the site-diretly modified *gyrB* mutated in position A136H (*gyr*B_A136H). According to literature this modification greatly increases (~4,6 times) the resistance of the purified enzyme in *in vitro* experiments. I wanted to examine this effect in *in vivo* circumstances. In pDTA_*synth* the *gyrB* was exchanged to artifitial *synth_gyrB* created with the overlapping mega-primer PCR technique. Finally, in the pDTA_*arr2* vector *gyrB* was exchanged for the *arr2* gene, which confers rifampicin resistance, using the same overlapping mega-primer PCR technique. All of the vectors were analysed by sequencing prior to further application.

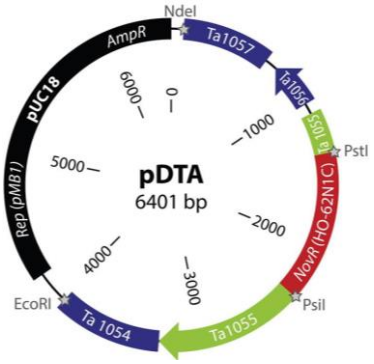

FIGURE 4. PDTA INTEGRATIVE VECTOR MAP

3.3.3 Linear constructs

In several cases of Archaea and Eukaryote, successful transformation was achieved by linear DNA constructs. Two genes were targeted by my knock-out (KO) constructs: Ta0895 – unknown protein but presumably coding an ubiquitin-like protein, and Ta1490 – tricorn protease. The KO constructs had three main elements: a resistance gene as core (red arrow), *synth_gyrB* and *arr2*, upstream (5' direction, green rectangle) and downstream (3' direction, blue rectangle) regions were homologous to the genome. **Figure 5.** illustrates the schematic map of the constructs with genomic target regions.

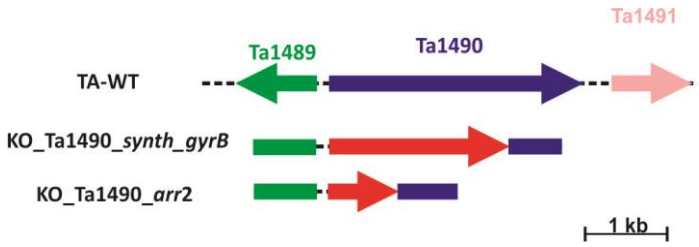

FIGURE 5. SCHEMATIC MAP OF LINEAR CONSTRUCTS DESIGNED FOR KNOCK-OUT MUTAGENESIS OF TA1490 WITH THE RELATED GENOMIC TARGET REGION

NEW SCIENTIFIC RESULT (III. thesis, based on results of 3.3. chapter): By the application of the selection marker genes (Nov^R *gyrB* and Rif^R *arr2*) shuttle (pSTA and pSTRif), integrative (pDTA, pNTA, pDTA_synth and pDTA_arr2) and linear vector constructs (KO_Ta1490_*synth_gyrB* and KO_Ta1490_*arr2*) were created for *Thermoplasma acidophilum*.

3.4 Development of new Archaea transformation method

One of the most critical steps for genetic manipulation is the transformation method by which nucleic acid can be introduced into the target organism. During my doctoral work I have tired to adapt electroporation, gene gun, lipofection, and magnetofection for transformation of *T. acidophilum* without success, that's why a completely new method had to be developed.

According to literature it is known that upon elevated pH *T. acidophilum* cells become plastic. Additionally, several Archaea are known to undergo natural from the environment. Main steps of the transformation method, which relied on this

information, are illustrated by **Figure 6.**. During transfromation positive growing controls were used to test if the cells survived the transformation or not, besides the negative control was adapted to examine the spontaneous mutations. After transformation and regeneration, 200 µl of cell culture were spread onto novobiocin- and rifampicin-containing Gelrite-based solid medium. With this method I could reach 10^2-10^4 transformants/µg DNA.

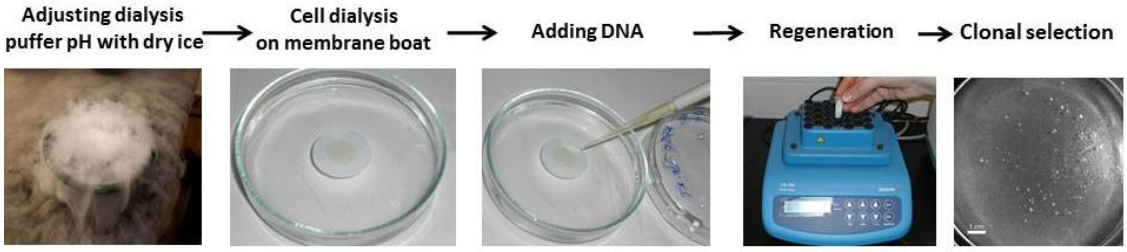

FIGURE 6. DIALYSIS-BASED TRANSFORMATION METHOD DEVELOPED FOR *T. ACIDOPHILUM*

NEW SCIENTIFIC RESULT (IV. thesis, based on results of 3.4. chapter): For introducing genetic material a new and specific transformation method has been developed for *Thermoplasma acidophilum*, reaching an efficacy of 10^2-10^4 transformant/µg DNA.

3.5 Analysis of resistant *T. acidophilum* cells

After transformation of *T. acidophilum*, transformants were inoculated in liquid and on solid media containing 100 ng/ml novobiocin or 2500 ng/ml rifampicin. The occurrence of the transformation was verified either by antibiotic resistance or molecular methods by which plasmid maintenance or genomic integration analysed in accordance with the vector constructs.

3.5.1 Novobiocin-resistant *T. acidophilum* cell lines

The novobiocin resistance of the following cell lines was tested: TA-WT, pDTA, pNTA, pDTA_*synth*. Results are illustrated in **Figure 7.** The wild type *T. acidophilum* (blue line) could not tolerate novobiocin even in low concentrations. The pDTA- (orange line) and pDTA_*synth*- (green line) harbouring cell lines tolerated 1500 ng/ml novobiocin. The pNTA-bearing cell lines coped with 2500 ng/ml novobiocin concentration. The pDTA vector carried the same mutant Nov^R *gyrB* gene as HO-62N1C. According to literature HO-62N1C tolerated 1000 ng/ml novobiocin . Since

my vector (pDTA) harboured the same *gyrB*, I expected that the resistance of the two stains would be identical. The increased resistance may be explained by the presumably higher amount of GyrB because transformants could contain the plasmid-localised mutant *gyrB* gene than the wild type cells. The pNTA cell line bore the modified Nov^{R} *gyrB* (A136H). According to literature this modification increases the resistance (~4,6 times) of the purified enzyme during *in vitro* experiments. So the experienced increase of resistance during *in vivo* experiments corresponded to the literature. The pDTA_*synth* vector was the same as pNTA on amino acid level, but the resistance was lower, likely due occurence of rare codons and thus lower amounts of expressed GyrB.

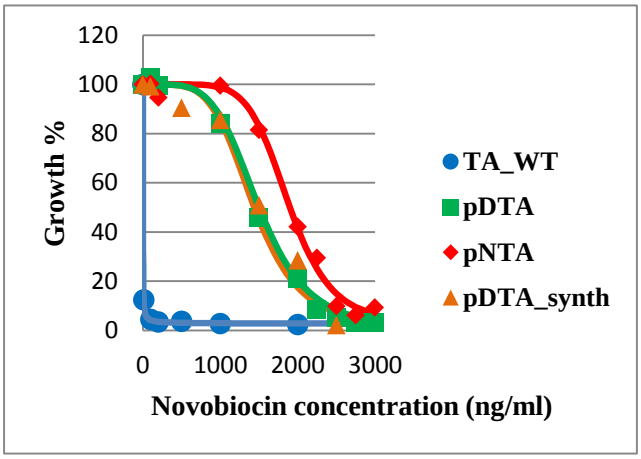

FIGURE 7: RESULTS OF NOVOBIOCIN RESISTANCE TEST OF NOVOBIOCIN RESISTANT CELL LINES

First the plasmid maintenance was analysed in the novobiocin-resistant cell lines. In contrary to my expectations I could not verify plasmid maintenance in *T. acidophilum* cells transformed with pSTA shuttle vector. In contrast to this, I surprisingly recognised that in some cases an intact or modified version of the pDTA integrative vector could be isolated from transformants and *E. coli* cells could be transformed with these isolates.

For investigation of integration, genomic DNA was isolated from novobiocin *T. acidophilum* cells. By primers of outside regions (up- and downstream) the target genomic region was amplified and subsequent nested PCR reaction sequence analysis of the target region was performed. In case of pSTA, pDTA and pNTA (carrying the corresponding resistant *gyrB*) harbouring cell lines, homologous recombination was

confirmed. Genbank accession numbers of the identified sequences are in case of pSTA: KC710298– KC710299 and in case of pDTA and pNTA: KC710300–KC710319. The *synth_gyrB* gene from the pDTA_*synth* could not be detected in the target region, but since cell lines were novobiocin-resistant I performed resistance gene (*synth_gyrB*) specific PCR reaction for the total genomic DNA, which was positive. So the resistance gene integrated unspecifically into the genome.

After transformation with linear KO constructs, novobiocin resistance of cell lines was confirmed but I experienced the same event as with pDTA_*synth*, that the linear constructs could not be detected at the target region and the resistance gene integrated randomly into the genome.

3.5.2 Rifampicin-resistant *T. acidophilum* cell lines

The rifampicin resistance of the following cell lines was tested: TA-WT, pDTA_*arr2*. Results are illustrated by **Figure 8.**. The wild type *T. acidophilum* (blue line) did not tolerate rifampicin even in low concentration as shown earlier . The pDTA_*arr2* vector (red line) harbouring cell lines was resistan, tolerated up to 2500 ng/ml rifampicin.

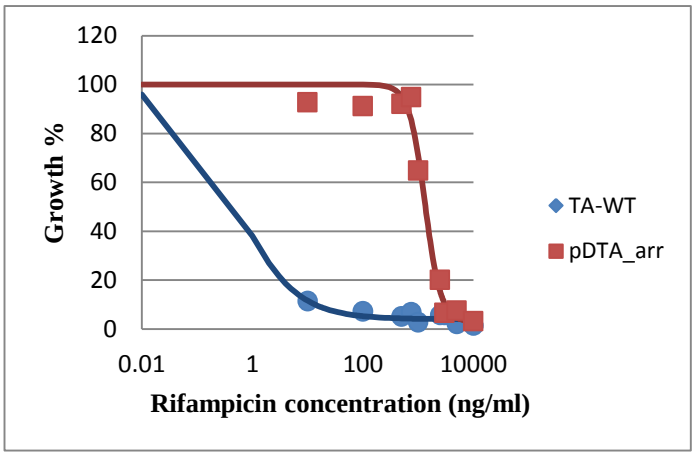

FIGURE 7. RESULTS OF RIFAMPICIN RESISTANCE OF RIFAMPICIN RESISTANT CELL LINE

Intact or a deletion variant of pSTRif was isolated from transformed *T. acidophilum* cells.

The genome integration os pDTA_*arr2* analysis was performed as described earlier. I could verify that homologous recombination did not occur in any cell line but the resistance gene integrated randomly into the genome.

Integration positions of KO_Ta1490_*arr2* linear construct transformed *T. acidophilum* were identified: Ta0060, Ta710 and Ta1490 C-terminal. Although in the latter case the construct integrated into the target gene, not matching with its intended target location. Genbank accession numbers of genomic integration: JX890289-JX890291.

The described results cearly indicate that the *T. acidophilum* DSM 1728 strain is capable of foreign DNA genomic integration via illegitimate recombination. The exploration of this mechanism might be a appractive research target.

NEW SCIENTIFIC RESULT (V. thesis, based on results of 3.5. chapter): The occurrence of transformation was verified by antibiotic resistance tests and molecular methods – plasmid maintenance tests and genomic integration analysis.

5. CONCLUSIONS AND SUGGESTIONS

Since its discovery Archaea gained a special role in research due to several special features. In order to study an organism in detail genetic manipulation tools are required. This is the only way to fully understand cellular processes and their relation, roles of proteins and subunit structures of protein-enzyme-complexes. *Thermoplasma acidophilum,* a thermo-acidophilic Archaeon was in the center of my work. This organism became a recognized model organism of proteomic studies due to resemblances to eukaryotic cells, and its low genome and proteome complexity. Additionally, cellular size and morphology make it ideal for cryo-electronmicroscopic studies. The missing genetic manipulation tools in this taxon greatly hamper the widening of proteomics and structural biology research; resolving this problem was the main aim of my doctoral work.

Hardly any preliminary research was done in this areas, two independent groups abandoned genetic research of *Thermoplasma* due to unforeseen challenges.

During development of genetic tools four parallel areas should be considered in parallel: clonal selection, identification of selection markers, building vector constructs and development of an effective transformation method.

As a first step cultivation of the organism including optimisation of solid cultivation as pre- requirement of clonal selection was solved. The organism was formerly cultivated on solid medium by Japanese researchers, but due to long incubation time (30 days) it was not suitable for routine clonal work. In order to increase the hardness of the Gelrite-based solid medium, I elevated the concentration of the solidification agent, making it more resistant against mechanical stress applied by i.e. streaking with inoculation loops. In parallel, I set up experiments to decrease the incubation time until visible colonies appeared so decreasing the generation time. The organism requires a complex medium, in which usually yeast extract provides the essential nutrients for the organism, however the means of extract preparation determines the growth promoting effect. In order to increase the growth rate, I increased the industrial yeast extract amount in the medium. Additionally, I prepared a yeast vitamin, which further enhanced cell growth greatly. Since cells during solid cultivation were extremely sensitive for temperature and humidity drops, solid

cultivation experiments were performed in a sealed jar. By these modifications I could develop an effective and routinely applicable solid medium on which *T. acidophilum* was able to form 1-3 mm in diameter colonies after 12 days of incubation, making the clonal selection feasible. Later I plan to investigate the yeast vitamin in detail to identify the growth promoting factor, earlier reported to be presumably of oligopeptidic nature.

Parallely to the cultivation experiments, several antibiotics were tested in order to find selection markers by which genetically modified cells could be separated from wild type cells. The organism has been reported to have sensitivity against few antibiotics like novobiocin, an aminocoumarin which inhibits growth in considerably low concentrations (10 ng/ml), and neomycin, which belong to the aminoglycosides. Besides that, erythromycin, chloramphenicol, coumermycin A, rifampicin, and thiostrepton were verified to inhibit the growth of the organism in the commonly applied concentration range. Suprisingly, rifampicin inhibited the growth of the organism in a rather low concentration (1000 ng/ml). This antibiotic is effective against Bacteria, but not Eukarya or Archaea. That DNA-dependent-RNA-polymerase of *T. acidophilum,* which resembles eukaryotic forms of this enzyme, was reported insensitivite as found in *in vitro* experiments during the eighties of the last century. However, my investigations clearly indicated that this does not hold true *in vivo*, as suggested in well-known prokaryotic taxonomy books (Bergey's Manual of Systematic Bacteriology, Prokaryotes). A specified antibiotic can only be used in a target organism if a resistance marker gene can be found, which confers resistance in the target organism. For novobiocin and rifampicin I found marker genes, with which I could confer resistance in *T. acidophilum*: a mutant gyraseB (*gyrB*-novobiocin) originating from a specific *T. acidophilum* strain and a rifampicin-ribosylase (*arr2*) originating from *Pseudomonas spp*. For advanced applications, such as performing multiple gene knock-outs simultaneously, identification of erythromycin, chloramphenicol and thiostrepton marker genes may provide a solution, though for my current work it was not required.

The most critical part of the genetic manipulation is the transformation, by which genetic material is introduced into the target organism. Since as of yet no transformation of *T. acidophilum* has been reported this seemed to be the hardest task of my work. Several physical, chemical and biological methods such as

electroporation, liposome-mediated and PEG-spheroplast transformation exist, by which other archaeal species are routinely transformed. During my work I tested a number of methods commonly used in archaeal genetics and when proven unsuccessful turned to further rarely used approaches such as gene gun transformation without reliable and reproducible success. Trailing these experiments, I developed a novel transformation method, taking advantage of the unique morphology of the organism (cell wall-less) and the observation that *T. acidophilum* cells become plastic at elevated pH. With the established protocol, I obtained between 10^2–10^4 transformants per µg DNA, which is a similar efficiency as reached with other archaeal transformation methods. A potential advantage of this method is that it presumably can be easily adapted to other members of the *Thermoplasmatales* order.

With the chosen selection marker genes, the mutant gyraseB (*gyrB*-novobiocin) and the rifampicin-ribolysase (*arr2*), vector constructs were built to test the newly developed transformation method. Novobiocin selection marker genes were a mutant gyrase (*gyrB*) gene and its modified version isolated from a novobiocin-resistant environmental strain (HO62N1C). Aim of the mutagenesis was to prove the increase of novobiocin resistance according to former studies with purified gyraseB. One of the most remarkable moments of my work was when I generated for the first time novobiocin-resistant *T. acidophilum* clones. The novobiocin resistance of the transformants, which carried the same *gyrB* as the mutant Japanase *T. acidophilum* environmental strain was 1500 ng/ml, a 1.5-fold increase in resistance compared to the mutant strain. Probably more than one copy of the Nov^R *gyrB* was expressed in the transformed cells, so that the total enzyme amount would be consequently higher. Cell lines harbouring the modified Nov^R *gyrB* (A136H) could tolerate a higher novobiocin concentration of up to 2500 ng/ml, which matches the reported data, that this amino acid position has a pronounced role in the antibiotic-enzyme interaction, making Nov^R *gyrB*_A136H is a prominent selection marker candidate for further research.

Over the course of investigations I experienced that the Nov^R *gyrB* gene of my vector constructs was capable to recombine into the genome of novobiocin-resistant cell lines, probably due to the high homology of the two genes. In order to direct this recombination, a selection marker incapable of spontaneous recombination was needed. For resolution of this problem a synthetic Nov^R *gyrB* was designed by codon

degeneration in such a way, that maximally 5 base pair long DNA stretches were identical with the original nucleotide sequence without altering the amino acid sequence. I generated novobiocin-resistant cell lines with integrative vectors, carrying this synthetized *gyrB* , and maintenance of the selection marker gene was proven. However, further analysis revealed, that the integration event was non-specific.

Parallely, an integrative vector harbouring Rif^R *arr2* was developed. This resistance marker has two main advantages. Firstly, resistance is realized by the inactivation of the antibiotic and not by blockage of the active site of the antibiotic target. Secondly, the *arr2* gene is short, which is a great benefit, as small vectors can hold longer target gene inserts. After transformation I could generate rifampicin-resistant cell lines tolerating up to 2500 ng/ml rifampicin. But I experienced similar random integration of the *arr2* gene as with pDTA_*synth*. These results suggest that in *T. acidophilum* DSM 1728 homologous recombination events occur next to illegitimate recombination events, though further exploration of this mechanism was not part of my aims.

In order to test plasmid maintenance in *T. acidophilum* I developed shuttle vectors, applying the ~16 kbp endogenous pTA1 plasmid isolated from the *T. acidophilum* HO-122 strain. A 6000 bp fragment of the pTA1 plasmid carrying the ori region was cloned into pRSF_Duet *E. coli* vector together with Nov^R and Rif^R selection marker genes. After transformation with *E. coli-T. acidophilum* shuttle vectors I could generate resistant (novobiocin and rifampicin) cell lines. But only pSTRif, harbouring *arr2,* could be recovered in considerably small amount. The smaller size of this shuttle vector compared to the ones carrying the novobiocin resistance genes might be the reason for its maintenance. Shuttle vectors were created for later protein overexpression purposes but due to the extremely low copy number these vectors could not be used for large-scale expression. In order to increase the copy number, reduction of size of the pTA1 fragment would be advisable, which would require identification of the currently unknown gene functions encoded by pTA1. EZ-transposon random mutagenesis seems to be the most suitable tool for functional mapping pf pTA1 plasmid and individual gene knock-outs of the 17 unknown genes encoded by pTA1. These plasmids should be transformed back into *T. acidophilum* and their copy number should be tracked. This work is already in progress.

The results of my work could initiate deeper genetic research of this model organism. Over the course of my PhD research time I could develop tools constituting a comprehensive genetic manipulation system. I could solve routine clonal selection of this microbe, developed an efficient and robust transformation method, and enabled usage of two different selection markers for transformant selection. This system or elements of it might be adapted to other species belonging to the *Thermoplasmatales* order which also currently lack genetic tools. Practical application of this system for special protein expression or knock-out still require further efforts, since illegitimate recombination events make it difficult to entirely control the locus of genetic modification. Application of other *T. acidophilum* strains not this countinously cultivated in the last thirty years but like those, recently added to the Japanese Collection of Microorganisms (JCM) might be a solution.

6. PUBLICATIONS

Scientific paper:

Baka E, Varga S, Hobel C, Knispel RW, Fekete Cs, Ivanics M, Kriszt B, Nagy I, Kukolya J (2013): The first transformation method for the thermo-acidophilic archaeon *Thermoplasma acidophilum*, Journal of Microbiological Methods, 95:145-148p, (IF 2012: 2.161)

Proceeding:

Baka E., Kriszt B., Jenes B., Ivanics M., Nagy I., R. W. Knispel, C. Hobel, Kukolya József (2010): Developing genetic tools for molecular biology analysis of the archaeon *Thermoplasma acidophilum*, TUDOC – Kárpát Medencei Doktoranduszok Nemzetközi Konferenciája, 2010. Május 27-28, Gödöllő, Konferencia kötet ISBN szám: 978-963-269-186-2, p

Communication published in Congress publications (concerning ISBN, ISSN or other, authenticated publications)

E. Baka, C. Fekete, S. Varga, RW. Knispel, I. Nagy, J. Kukolya (2013): Development of basic genetic manipulation system for the thermoacidophil *Thermoplasma acidophilum*. Hungarian Life Science Conference, Book of abstracts, p 104-105. ISBN Number: 978-615-5270-02-4

E. Baka, S. Varga, Cs. Fekete, Á. Hubert, I. Nagy, J. Kukolya (2013): Plasmid isolation from *Thermoplasma acidophilum* HO-122 for shuttle vector construction. Acta Microbiologica et Immunologica Hungarica, 60: 111-112.

S. Varga, **E. Baka**, B. Kriszt, S. Szoboszlay, RW. Knispel, W. Baumeister, I. Nagy, J. Kukolya (2012): New approaches in the development of genetic tools for *Thermoplasma acidophilum*. Acta Microbiologica et Immunologica Hungarica, 60: 106.

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Baka E., Kriszt B., Jenes B., Ivanics M., Nagy I., Kukolya J. (2009): Developing of genetic tools for the model organism, *Thermoplasma acidophilum*. Acta Microbiologica et Immunologica, 56: 118-119.

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